ANTISENSE OLIGONUCLEOTIDES CAPABLE OF INHIBITING THE FORMATION OF CAPILLARY TUBES BY ENDOTHELIAL CELLS

Related Application

[0001] This is a continuation of International Application No. PCT/FR02/02067, with an international filing date of June 14, 2002, which is based on French Patent Application No. 01/07805, filed July 14, 2001.

Field of the Invention

[0002] This invention relates to antisense oligonucleotides capable of inhibiting the expression of the protein IRS-1 and inhibiting the formation of capillary tubes by endothelial cells. Thus, the invention relates to antiangiogenic agents and anti-cell-multiplication agents, particularly, antitumor agents. The invention also pertains to pharmaceutical compositions containing said oligonucleotides and the use of said oligonucleotides as analysis reagents.

Background

[0003] Angiogenesis is a fundamental process by means of which new blood vessels are formed. This process is essential in multiple normal physiological phenomena such as reproduction, development and even cicatrization. In these normal biological phenomena, angiogenesis is under strict control, i.e., it is triggered during a short period (several days) and then completely inhibited. However, many pathologies are linked to uncontrolled, invasive angiogenesis: arthritis, a pathology due to the damaging of cartilage by invasive neovessels; diabetic retinopathy or the invasion of the retina by neovessels leading to blindness of patients; neovascularization of the ocular apparatus which is a major cause of blindness. This

neovascularization is involved in about twenty different eye diseases. Moreover, the growth and metastasis of tumors which are linked directly to neovascularization are dependent on angiogenesis. The tumor stimulates the growth of neovessels by its own growth. Moreover, these neovessels are escape routes for tumors which thereby join up with the blood circulation and induce metastases in sites remote from the initial tumor focus, such as the liver, lungs or bones.

[0004] Angiogenesis, the formation of neovessels by endothelial cells, involves the migration, growth and differentiation of endothelial cells. Regulation of these biological phenomena is directly linked to genetic expression.

Summary of the Invention

[0005] This invention relates to a pharmaceutical composition that blocks angiogenesis including as active agent at least one substance selected from the group consisting of (i) a nucleic acid molecule of a gene coding for protein IRS-1, a complementary sequence or a fragment thereof and (ii) a molecule which inhibits expression of a nucleic acid molecule according to (i).

[0006] This invention also relates to a method of inhibiting angiogenesis including administering a pharmaceutically effective amount of the pharmaceutical composition.

[0007] This invention further relates to a method of treating retinopathy, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis associated with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular diseased, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infraction, chronic heart disease, congestive heart failure, age-related macular degeneration or

osteoporosis including administering a pharmaceutically effective amount of the pharmaceutical composition.

[0008] This invention still further relates to a method of diagnosing pathologies linked to angiogenesis including contacting a composition containing an active agent including at least one substance selected from the group consisting of (i) a nucleic acid molecule of a gene coding for protein IRS-1, a complementary sequence or a fragment thereof and (ii) a molecule which inhibits expression of a nucleic acid molecule according to (i) and target cells in a condition sufficient to permit inhibition of IRS-1 gene expression; measuring expression of the IRS-1 protein by the cells; and comparing expression of the protein measured before and after hybridization to measure inhibition of the expression.

Brief Description of the Drawings

[0009] Other advantages and characteristics of the invention will become clear from the examples below in which the term "oligonucleotide" is used to designate the oligonucleotide of SEQ ID NO. 3 and which refer to the attached figures in which:

Fig. 1A is a Western Blot of images obtained from supernatant samples stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track S) developed with an anti-IRS-1 antibody,

Fig. 1B is a Western Blot of images obtained after staining with silver nitrate obtained from the same supernatant samples stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track S),

Fig. 2 is a Western Blot of images obtained from supernatant cells stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track B) when the membrane is

incubated with an anti-phosphotyrosine monoclonal antibody and developed with an anti-isotope antibody tagged at the peroxidase as indicated in Example 3,

Figs. 3A to 3D show the images of the cultures on a type I collagen surface of the different lots of endothelial cells:

Fig. 3A shows the culture of untreated endothelial cells,

Fig. 3B shows the culture of endothelial cells stimulated with 3 ng/ml of bFGF,

Fig. 3C shows the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours and then stimulated with 3 ng/ml of bFGF,

Fig. 3D shows the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours,

Figs. 4A to 4F illustrate the results of tests of the inhibition of corneal neovascularization in rats:

Fig. 4A shows the results obtained by subconjunctival injection of an antisense oligonucleotide at a concentration of 60 µm,

Fig. 4B shows the results obtained after subconjunctival injection of a sense oligonucleotide at a concentration of 60 µm,

Fig. 4C shows the results obtained after topical application of an antisense oligonucleotide at a concentration of 200 µm,

Fig. 4D shows the results obtained after topical application of a sense oligonucleotide at a concentration of 200 µm,

Fig. 4E illustrates the state of the cornea in the absence of any treatment,

Fig. 4F illustrates the state of the cornea when treated with subconjunctival injections of PBS,

Figs. 5A to 5J illustrate the results of the inhibition of corneal neovascularization obtained in different groups of rats after de-epithelialization and limbic resection of the corneas of the rats on day 4 (Figs. 5A to 5E) and on day 9 (Figs. 5F to 5J). These are slit lamp photographs showing the comparison of the growth of the vessels in the various groups of rats. Enlargement x10.

Detailed Description

[00010] Our work performed in the framework of this invention made it possible to identify and prepare nucleic acid sequences involved in the regulation of angiogenesis.

[0010] Other studies pertaining to angiogenesis have shown a noteworthy expression and phosphorylation at the level of a tyrosine residue of an intracellular 180-kDa protein by endothelial cells cultured on a surface of type I collagen and stimulated by an angiogenic factor such as bFGF. The noteworthy expression and phosphorylation at the level of the tyrosine residue of the intracellular 180-kDa protein accompanies the formation of capillary tubes by the endothelial cells.

[0011] That protein is already known as a substrate of the insulin receptor (called IRS-1). It has been partially identified and investigated by certain diabetes researchers (Quon et al., J. Biol. Chem. (1994), 269 (45), 27920-27924). Those authors studied the role of IRS-1 in (i) the translocation of GLUT 4 stimulated by insulin and (ii) the transport of glucose in rat adipose cells. In order to do this, they constructed a plasmid containing:

– a double chain oligonucleotide obtained from the sense oligonucleotide of the following sequence SEQ ID NO. ID No. 1: 5'-TCGATGTGAC GCTACTGATG AGTCCGTGAG GACGAAACTC TGGCCTAG-3' and

– cDNA coding for human IRS-1,

and transfected rat adipose cells with said plasmid.

[0012] Our work revealed that the expression of the protein IRS-1 is also induced in endothelial cells when those cells are stimulated by the angiogenic factor bFGF.

[0013] The invention thus pertains to a pharmaceutical composition active on angiogenesis phenomena comprising as active agent at least one substance selected from among: (i) a nucleic acid molecule of the gene coding for the protein IRS-1, a complementary sequence or a fragment thereof, (ii) a molecule which inhibits the expression of a nucleic acid molecule according to (i).

[0014] In the framework of the invention, antisense oligonucleotides of the gene coding for this protein were prepared. These oligonucleotides have remarkable antiangiogenic and antitumor activities. They are therefore particularly useful in the treatment of diseases linked to invasive angiogenesis not controlled by gene therapy methods including administering to an individual a composition containing at least one of these oligonucleotides.

[0015] Thus, an oligonucleotide according to the invention is constituted by the following nucleotide sequence of formula SEQ ID NO. 2:

5-TATCCGGAGGGCTCGCCATGCTGCTGCGGAGCAGA-3',

a fragment thereof comprising at least 12 contiguous nucleotides or their derivative.

[0016] The invention pertains most particularly to an oligonucleotide constituted by one of the nucleotide sequences of formulas SEQ ID NO. 3 and 4 below:

5'-TATCCGGAGGGCTCGCCATGCTGCT-3',

5'-TCGCCATGCTGCTGCGGAGCAGA-3',

a fragment of these comprising at least 12 contiguous nucleotides or their derivative.

[0017] The term "derivative" is understood to mean a sequence capable of hybridizing under strict conditions with one of the sequences SEQ ID NO. 2, 3 or 4, or with a fragment of these of at least 12 contiguous nucleotides.

[0018] The following sequences can be cited as non-limiting examples of oligonucleotides according to the invention:

SEQ ID NO. 5: 5'-TATCCGGAGGGCCTGCCATGCTGCT-3',

SEQ ID NO. 6: 5'-TATCCGGAGG GCCTGCCATG CTGC-3',

SEQ ID NO. 7: 5'-TATCCGGAGG GCCTGCCATG CTG-3',

SEQ ID NO. 8: 5'-TATCCGGAGG GCCTGCCATG CT-3',

SEQ ID NO. 9: 5'-TATCCGGAGG GCCTGCCATG C-3',

SEQ ID NO. 10: 5'-TATCCGGAGG GCCTGCCATG-3',

SEQ ID NO. 11: 5'-TATCCGGAGG GCCTGCCAT-3',

SEQ ID NO. 12: 5'-TATCCGGAGG GCCTGCCA-3',

SEQ ID NO. 13: 5'-TATCCGGAGG GCCTGCC-3',

SEQ ID NO. 14: 5'-TATCCGGAGG GCCTGC-3',

SEQ ID NO. 15: 5'-TATCCGGAGG GCCTG-3',

SEQ ID NO. 16: 5'-TATCCGGAGG GCCT-3',

SEQ ID NO. 17: 5'-TATCCGGAGG GCC-3',

SEQ ID NO. 18: 5'-TATCCGGAGG GC-3',

SEQ ID NO. 19: 5'-CCGGAGG GCCTGCCATG CTGCT-3',

SEQ ID NO. 20: 5'-GAGG GCCTGCCATG CTGCT-3',

SEQ ID NO. 21: 5'-G GCCTGCCATG CTGCT-3',

SEQ ID NO. 22: 5'-CTGCCATG CTGCT-3'.

SEQ ID NO. 23: 5'-TGCCATG CTGCT-3'.

[0019] All or part of the phosphodiester bonds of the invention are advantageously protected. This protection is generally implemented via the chemical route using methods that are known by art. The phosphodiester bonds can be protected, for example, by a thiol or amine functional group or by a phenyl group.

[0020] The 5'- and/or 3'- ends of the oligonucleotides of the invention are also advantageously protected, for example, using the technique described above for protecting the phosphodiester bonds.

[0021] The oligonucleotides of the invention can be synthesized using conventional techniques that are known art, for example, using one of the DNA synthesizers marketed by various companies.

[0022] Although their mechanism of action has not been entirely elucidated, the oligonucleotides according to the invention inhibit the expression of the protein IRS-1 within endothelial cells. These oligonucleotides block the formation of neovessels by endothelial cells (i.e., they inhibit angiogenesis) and thus they inhibit the multiplication of tumor cells in mice.

[0023] The invention therefore also includes a pharmaceutical composition that inhibits the gene coding for the protein IRS-1 comprising at least one oligonucleotide complementary of a part of said gene or of a transcript of said gene.

[0024] The molecule capable of inhibiting the expression of a nucleic acid molecule of the gene coding for the protein IRS-1 is preferably an antisense sequence of the region coding the sequence identified under the number SEQ ID NO. 28. The antisense sequence advantageously comprises at least twelve contiguous nucleotides or their derivative.

[0025] More preferentially, the active agent capable of inhibiting the expression of a nucleic acid molecule coding for the protein IRS-1 of the composition of the invention is a

nucleotide sequence selected from SEQ ID NO. 2 to SEQ ID NO. 23 comprising at least twelve contiguous nucleotides or their derivative.

[0026] Such a composition advantageously comprises as an active agent at least one oligonucleotide as defined above advantageously combined in said composition with an acceptable vehicle.

[0027] The research performed in the framework of the invention made it possible to demonstrate that the protein IRS-1 represents a cellular constituent which is essential in the angiogenesis process. In fact, inhibition of the expression of the protein IRS-1 by said antisense oligonucleotides leads to the inhibition of the formation of capillary tubes by endothelial cells.

[0028] The oligonucleotides according to the invention and the compositions containing them are thus antiangiogenic agents. They are also anti-cell-multiplication agents, particularly as antitumor agents, and consequently are particularly useful for the treatment of tumors. Thus, the invention includes the use of said oligonucleotides for the preparation of a composition intended for the treatment or prevention of pathologies linked to invasive, uncontrolled angiogenesis such as, as a nonlimitative example: the treatment of tumor vascularization, eye diseases linked to the neovascularization of the ocular apparatus such as retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis associated with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure, age-related macular degeneration and osteoporosis.

[0029] The above pharmaceutical compositions are more particularly implemented in a manner such that they can be administered via the subcutaneous, intramuscular, intravenous or transdermal route, for example. For such administration, use is made of aqueous suspensions, isotonic saline solutions or sterile, injectable solutions containing pharmacologically compatible dispersion agents and/or wetting agents such as, for example, propylene glycol or butylene glycol.

[0030] The usual unit dose to be administered contains from about 0.001 mg to about 50 mg of active principle.

[0031] The oligonucleotides of the invention are also useful as research reagents, notably for the *in vitro* study of signalization routes involving the 180-kDa protein, for example, on tumor cells or non-tumor cells transfected by the oligonucleotides. They are also useful for the *in vivo* study of signalization routes involving the 180-kDa protein in a large number of physiological and pathological phenomena such as angiogenesis or carcinogenesis essentially from the kinase/phosphatase ratio.

[0032] Thus, the pharmaceutical compositions of the invention are particularly useful for the performance of tests for the diagnosis of pathologies linked to angiogenesis phenomena, notably for the diagnosis of retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis association with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure or age-linked macular degeneration and osteoporosis.

[0033] According to preferred aspects of the invention, the method of diagnosis comprises the follow steps:

contacting a composition containing an active agent including at least one substance selected from the group consisting of (i) a nucleic acid molecule of a gene coding for protein IRS-1, a complementary sequence or a fragment thereof and (ii) a molecule which inhibits expression of a nucleic acid molecule according to (i) and target cells in a condition sufficient to permit inhibition of IRS-1 gene expression;

measuring expression of the IRS-1 protein by the cells; and

comparing expression of the protein measured before and after hybridization to measure inhibition of the expression.

The condition sufficient to permit inhibition of the IRS-1 gene expression are known in the art. Measuring the expression of the IRS-1 protein may be performed by techniques known in the art such as, for example, recognition by antibodies.

Example 1: Demonstration of the induction of the expression of IRS-1 (the 180-kDa protein) in endothelial cells resulting from the stimulation of these cells with bFGF.

[0034] The 180-kDa protein was demonstrated in the following manner:

The endothelial cells were cultured in a 6-well microtitration plate previously covered with type I collagen as described in (Montesano et al., J. Cell. Biol., 1983, 83, 1648-1652). The culture medium was DMEM (Sigma) enriched with 10% of fetal calf serum, 4 mM glutamine, 500 U/ml penicillin and 100 µg/ml streptomycin. After 3 to 4 days of culture, there resulted a semi-confluent layer of endothelial cells. The culture medium of six wells was aspirated and replaced by fresh culture medium. Three wells were enriched with 3 ng/ml of bFGF. After incubation for 48 hours, the wells were washed three times with a phosphate buffer and the cells were used to extract the messenger RNA (mRNA) according to protocols known in the art. The

mRNAs were reverse transcribed by a polymerization chain reaction (PCR) using each of four degenerated groups of oligo (dT) (T12MN) primers, M can be G, A or C; and N is G, A, T and C. Each group of primers is imposed by the base in position 3'(N) with a degeneration in the (M) position. Example: the set of primers in which N = G is constituted by:

SEQ ID NO. 24: 5'-TTTTTTTTTTTGG-3'

SEQ ID NO. 25: 5'-TTTTTTTTTTTAG-3'

SEQ ID NO. 26: 5'-TTTTTTTTTTTCG-3'.

[0035] The cDNAs obtained in this manner were amplified and tagged by means of an arbitrary decamer in the presence of isotopically tagged ATP. The electrophoresis analysis of the cDNAs revealed the presence of an amplified 326-bp cDNA fragment in the sample stemming from the endothelial cells stimulated with bFGF, identified in the attached sequence listing as number SEQ ID NO. 27. However, this same fragment is weakly present or present in the trace state in the sample stemming from the endothelial cells that were not stimulated with bFGF. The sequencing of this fragment and the subsequent interrogation of the databases revealed that this fragment corresponds to a part of an already known gene, coding for the substrate of the insulin receptor (an intracellular 180-kDa protein).

Example 2: Demonstration of the induction of the expression of IRS-1 (the 180-kDa protein).

[0036] Endothelial cells cultured on a layer of type I collagen stimulated or not stimulated with bFGF (cf. example 1) were lysed in a cellular lyse buffer containing sodium orthovanadate. These solutions were then clarified by centrifugation at 14,000 g for 15 minutes. Supernatant samples stemming from unstimulated cells and cells stimulated with bFGF containing equivalent amounts of proteins were then taken up with an electrophoresis solution containing 2% SDS and 15 mM of dithiothreitol, heated at 100°C for 5 minutes then deposited on polyacrylamide gel (gradient from 4 to 15% of acrylamide) under denatured conditions (in the

presence of 2% SDS). After migration, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked by incubation at ambient temperature in a 5% milk solution in a PBS buffer. The membrane was then washed three times with a PBS buffer, incubated in a PBS buffer containing 1 μg/ml of anti-IRS-1 monoclonal antibody for 2 hours at ambient temperature and washed three times with a PBS buffer. The proteins were then developed with a secondary anti-isotope antibody coupled to peroxidase. The presence was noted of a protein of molecular weight 180 kDa recognized by the monoclonal anti-IRS-1 antibody in the preparations stemming from the endothelial cells stimulated with bFGF; this protein was weakly present in the preparation stemming from the endothelial cells not simulated with bFGF (Fig. 1).

Example 3: Demonstration of the induction of phosphorylation at the level of IRS-1 tyrosine (the 180-kDa protein).

[0037] Human endothelial cells cultured on a layer of type I collagen stimulated or not stimulated with bFGF were lysed in a cellular lyse buffer containing sodium orthovanadate. These solutions were then clarified by centrifugation at 14,000 g for 15 minutes (cf. example 2). The IRS-1 protein was extracted by means of an anti-IRS-1 monoclonal antibody. This extraction was performed after immunoprecipitation by means of an anti-IRS-1 monoclonal antibody (Sigma). After addition of the anti-IRS-1 antibody coupled to agarose, the suspension was incubated for 2 hours at ambient temperature then centrifuged at 4000 g for 15 minutes. The resultant precipitate was taken up with an electrophoresis solution containing 2% SDS and 15 mM of dithiothreitol, heated at 100°C for 5 minutes, then deposited on polyacrylamide gel (acrylamide gradient of 4 to 15%) under denaturing conditions (in the presence of 2% SDS). After migration, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked by incubation at ambient temperature in a 5% milk solution in a PBS buffer. The

membrane was then washed three times with a PBS buffer, incubated in a PBS buffer containing 1 μg/ml of anti-phosphotyrosine monoclonal antibody for 2 hours at ambient temperature, and then washed three times with a PBS buffer. The proteins were then developed by means of a secondary anti-isotope antibody coupled to peroxidase. It was found that the IRS-1 protein of molecular weight 180 kDa was phosphorylated at the level of the tyrosine residue in the preparations stemming from the endothelial cells stimulated with bFGF; this protein was very weakly phosphorylated at the level of the tyrosine residue in the preparation stemming from the endothelial cells not stimulated with bFGF (Fig. 2).

Example 4: Evaluation of the in vitro antiangiogenic activity of the oligonucleotide.

[0038] Human endothelial cells were cultured on a layer of type I collagen. The culture wells were divided into four lots on the seventh day of culture:

Lot 1: Wells corresponding to the culture of untreated endothelial cells (Fig. 3A).

Lot 2: Wells corresponding to the culture of endothelial cells stimulated with 3 ng/ml of bFGF (Fig. 3B).

Lot 3: Wells corresponding to the culture of endothelial cells incubated with 100 μg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours then stimulated with 3 ng/ml of bFGF (Fig. 3C).

Lot 4: Wells corresponding to the culture of endothelial cells incubated with 100 μg/ml of oligonucleotide of sequence SEQ ID NO. 3 for 4 hours (Fig. 3D).

[0039] The various wells were examined by means of an inverted phase optical microscope after 3 to 4 days of culture. Upon reading the results, it was found that the human endothelial cells in lot 2 formed capillary tubes following stimulation with bFGF. It was also found that the oligonucleotide inhibits the formation of neovessels by these same cells stimulated with bFGF in lot 3. Finally, it was found that that the oligonucleotide does not modify in a

pronounced manner the growth of the endothelial cells. In fact, the numbers of endothelial cells in the lot 1 wells and in the lot 4 wells were comparable.

Example 5: Evaluation of the in vivo activity of the oligonucleotide.

[0040] Three lots of naked mice were used. Each lot was constituted by 5 mice.

[0041] Lot no. 1: This lot was used as control. Each mouse was inoculated on day 0 with 200 µl of a suspension of B16 melanoma cells (provided by Institut Gustave Roussy, Villejuif) dispersed in PBS at the level of 10^6 cells/ml. These mice did not receive subsequent treatment.

[0042] Lot no. 2: Each mouse was inoculated subcutaneously on day 0 with 200 µl of a suspension of B16 melanoma cells dispersed in PBS at the level of 10^6 cells/ml. On day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 8, day 9 and day 10 each mouse received a subcutaneous injection of 200 µl of an oligonucleotide solution diluted in PBS at a concentration of 500 µg/ml. The oligonucleotide injection was performed close to the cell injection site.

[0043] Lot no. 3: The mice of this lot were not inoculated with the B16 melanoma cells. However, each of the mice received an injection of 200 µl of an oligonucleotide solution in PBS at a concentration of 500 µg/ml; the injections were performed on day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 8, day 9 and day 10.

[0044] The following results were obtained:

[0045] In the mice of lot no. 1, the tumor mass developed very rapidly after inoculation. In fact, the tumor mass reached a size of 1.6 to 2.5 cm in diameter after ten days in the mice of said lot no. 1 (untreated mice). The evolution of the tumor mass in the mice of lot no. 2 (mice treated after inoculation by injection of oligonucleotide on day 1, day 2 and day 3), exhibited a clearly lower increase in the volume of the tumor mass. The tumor mass in the mice of lot 2 did

not exceed 0.8 cm in diameter on the tenth day. On the fourteenth day, the difference between the tumor mass of the mice of lot no. 2 and those of lot no. 1 was remarkable.

[0046] In the mice of lot no. 3 (mice not having received B16 melanoma cells but treated by injection of oligonucleotide for three days), an unexpected general effect was observed on the skin. It was identical to that observed on all of the mice treated with the oligonucleotide (lot 2). The skin had an aged, crumpled appearance. The emergence of hairs was also observed on all of the treated mice. There was a parallelism during the evolution between the regression of the cutaneous signs and the resumption of tumor growth.

[0047] Thus, it was found that the oligonucleotide inhibits the development and formation of neovessels by endothelial cells *in vitro*. The oligonucleotide also has a remarkable *in vivo* antitumor activity in the naked mouse.

Example 6: Evaluation of the antiangiogenic oligonucleotide on a corneal neovascularization model in the rat.

[0048] We employed, modified and analyzed a model of the formation of corneal neovessels in the rat after de-epithelialization and limbectomy (Figs. 5A to 5J). It is reproducible, allows direct slit-lamp examination and quantification of the neovessels. The details are described below. The model was then used for testing the efficacy of the antiangiogenic agents of the invention.

Animals and corneal neovascularization model

[0049] Male Wister rats (*Rattus norvegicus*), aged five weeks (Charles River France, St-Aubin les Elbeufs, France), free of specific pathogens, were fed and allowed to drink water freely, and maintained in the laboratory animal facility under fixed temperature and humidity conditions, with cycles of 12 hours of light/12 hours of darkness.

[0050] The rats were anesthetized with a mixture of ketamine (Kétamine 1000, UVA, Ivry-sur-Seine, France; 128 mg/kg) and chlorpromazine (Largactil 25 mg/ml; Specia Rhône Poulenc, Paris, France; 5 mg/kg), injected via the intramuscular route. A drop of oxybuprocaine (Novésine, Chibret, Clermont-Ferrand, France) was instilled in the right eye. Using an enlargement system (macroscope Wild MPS 51 S, LEICA, Heerbrugg, Switzerland), the corneal epithelium was removed by a microsponge impregnated with 70% ethanol. A 1.5-mm band of conjunctiva, at the limbus, was excised with microsurgical scissors, and the eyelids were closed by a temporary blepharorraphy with a Vicryl 5.0 thread (Dacron, Alcon, Rueil-Malmaison, France). The eye was then rinsed abundantly with 1X PBS, an oxytetracycline cream was applied (Posicycline, Alcon, France) and the blepharorraphy was opened on the fourth day [8, 9].

Treatment by subconjunctival injections and topical applications of antiangiogenic oligonucleotide

[0051] The rats were divided into 6 groups:

Group A: model + subconjunctival injection of a 60-μM antisense oligonucleotide solution in 1X PBS,

Group B: model + topical application of a 200-μM antisense oligonucleotide solution in 1X PBS,

Group C: model + subconjunctival injection of a 60-μM sense oligonucleotide solution in 1X PBS,

Group D: model + topical application of a 200-μM sense oligonucleotide solution in 1X PBS,

Group E: model + subcutaneous injection of 1X PBS,

Group F: model without treatment.

[0052] All of the rats were subjected to de-epithelialization as described above; the treatment was performed every 24 hours starting on the fourth day and continuing until the ninth day. Neovascularization was examined at the beginning, in the middle and at the end of the protocol by slit-lamp examination; photographs were taken on day 0 and day 9.

Visualization and quantification of the neovascularization

[0053] The animals were euthanized 10 days after the de-epithelialization by lethal injection of pentobarbital (intraperitoneal injection). In order to fill the microvessels and quantify the corneal neovascularization, the upper part of the animals' bodies were perfused with fluorescein-dextran 2x1,000,000. The eyes were enucleated and immersed in paraformaldehyde/1X PBS 4% for 3 hours, then overnight in 1X PBS. The cornea was then isolated with 1 mm of limbus under surgical microscope and inserted in the flat state between plate and cover by means of 3 to 5 radial incisions. The flat corneas were then examined and photographed using fluorescence microscopy. After the whole corneas were reconstituted, they were scanned and the surfaces were measured by image analysis; a software program (NIH image) was used for the quantification of the neovascularization. For each photo, the total corneal surface was measured three times as was the neovascularized surface; the ratio of the means – neovascularized surface/total corneal surface – was used to obtain the percentage of neovascularization and to measure the inhibition obtained.

Statistical analysis

[0054] The results were expressed as means ± SD. The percentages of neovascularized surface/total surface were compared with the nonparametric test of Mann-Whitney. Values of $P < 0.05$ were considered to be significant.

Dilution of the oligonucleotide

[0055] The oligonucleotide was diluted in 1X PBS at pH 7.2. Based on the data in the literature and the experiments performed with other oligonucleotides, it was decided to use a concentration of 60 μM for the subconjunctival injections and a concentration of 200 μM for the topical applications.

Results

[0056] Using the model of corneal neovessels, treatment was performed with the 5'-TATCCGGAGGGCTCGCCATGCTGCT-3' oligonucleotides identified under SEQ ID NO. 3 in the attached sequence listing modified in phosphorothioate form, daily, from day 4 to day 9, according to the following protocol:

Group A: subconjunctival injection of the antisense oligonucleotide at 60 μM (AS 60),

Group B: topical application of the antisense oligonucleotide at 200 μM (AS 200),

Group C: subconjunctival injection of the sense oligonucleotide at 60 μM (S 60),

Group D: topical application of the sense oligonucleotide at 200 μM (S 200),

Group E: subconjunctival injection of 1X PBS (PBS),

Group F: no treatment (0 Tt).

[0057] On the tenth day of the protocol, the rats were perfused with a solution of FITC/dextran and then euthanized. The corneas were collected and fixed in a 4% PAF solution. The corneas were then inserted in the flat state between plate and cover in a glycerol solution. The fluorescent neovessels were observed and photographed using the fluorescence microscope. The photographs were scanned and the neovascularization percentages were measured for each animal.

[0058] The results observed are presented in Table 1 below:

Table 1

	Group A AS 60	Group B AS 200	Group C S 60	Group D S 200	Group E PBS	Group F 0 Tt
Mean	0.6157	0.5058	0.9431	0.9392	0.9552	9.9170
SD	0.2194	0.1172	0.0964	0.0308	0.0481	0.0751
Number of measurements	15	15	15	12	9	9
SEM	0.0566	0.0303	0.0249	0.0089	0.0160	0.0250

[0059] The statistical analysis of the results using a nonparametric Mann-Whitney test yielded the following results:

The subconjunctival injections of 60-μM of the antisense oligonucleotide (A) reduced neovascularization in relation to the control groups E and F (very significant results, $P < 0.0001$ and $P = 0.0011$); topical application of the antisense oligonucleotide at a concentration of 200 μM (B) reduced neovascularization in relation to the control groups E and F (extremely significant results, $P < 0.0001$).

[0060] Compared to the subconjunctival administration of the sense oligonucleotide at 60 μM (C) or the topical application of the sense oligonucleotide at 200 μM (D), injection of the antisense oligonucleotide at 60 μM (A) and topical application of the antisense oligonucleotide at 200 μM (B) reduced neovascularization. These results were extremely significant ($P < 0.0001$) (Figs. 4A to 4F).

[0061] The inhibition of neovascularization was not significantly different depending on whether the antisense oligonucleotide was administered via the subconjunctival route (60 μM) or applied topically (200 μM). It was approximately 35% in relation to the controls (E and F).

[0062] The subconjunctival injection of the sense oligonucleotide at 60 μM (C) and the topical application of the sense oligonucleotide at a concentration of 200 μM (D) did not modify the neovascularization in relation to the control groups (E and F). In contrast, there was a small

effect of the sense oligonucleotide in topical application (D) compared to the sense oligonucleotide in subconjunctival injections (C) (P = 0.0117).

[0063] Moreover, there was seen in the groups treated with the antisense oligonucleotide (A and B), a smaller diameter and density of the neovessels. Their distribution did not differ in relation to the control groups nor was any difference observed in relation to the level of inflammation (Fig. 4).

Secondary effects

[0064] No noteworthy secondary effects were seen in any of the groups during the two experimental series: after 6 days of treatment at the doses specified above, the skin of the rats was not crumpled, the fur was unchanged and the general condition of the animals was good; they fed normally until the last day and no suspicious mortality was observed. Although neither autopsies nor blood tests were performed, the general status of the animals at the end of the experiments did not suggest hepatic disorders. The only symptom observed was a transitory whitish deposit at the site of the conjunctival injections in 60% of the rats of group A, 60% of the rats of group C and 10% of the rats of group E. This deposit had been resorbed by the end of the experiments in all cases.

[0065] This example shows that — contrary to expectations — the subcutaneous injections of antisense oligonucleotide at a concentration of 60 μM did not inhibit neovascularization to a greater extent than the topical application of the antisense oligonucleotide at a concentration of 200 μM.

[0066] This can perhaps be explained by the difference in the concentrations employed; but this results suggests also a penetration of the oligonucleotide via the topical route rather than via the limbus. It also suggests the absence of prolonged release of the product from the injection site.

Conclusion

[0067] The application of the antisense oligonucleotide via the topical route or in subconjunctival injections reduces neovascularization in our model of corneal neovessels in the rat.

[0068] The purpose of this study was to test the efficacy of the antisense oligonucleotides stemming from the sequence of the gene IRS-1 on a previously developed model of corneal neovascularization in the rat.

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